PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 90/08829
C12N 15/82, A01H 5/00	A1	(43) International Publication Date:	9 August 1990 (09.08.90)

(21) International Application Number: PCT/GB90/00102

(22) International Filing Date: 25 January 1990 (25.01.90)

(30) Priority data:
8901674.5 26 January 1989 (26.01.89) GB

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: REGULATION OF PLANT GENE EXPRESSION

(57) Abstract

A recombinant plant gene for regulation of gene expression has a repressor protein gene of bacterial origin associated with an operator which is recognised by the bacterial repressor protein and which controls expression of a foreign structural gene.

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REGULATION OF PLANT GENE EXPRESSION

This invention relates to the regulation of plant gene expression. More particularly, the invention is concerned with the regulation of plant gene expression by the use of repressor molecules. The invention also provides materials for use as repressors and methods for the production thereof.

Traditionally, the improvement of crop plant species involves the introduction of desired traits by genetic crosses. However, although these breeding techniques are highly successful, they provide no means of controlling the expression of the newly acquired traits. Recent advances in technology are now allowing the genes responsible for determining plant structure and the productivity and quality of the crop to be identified and isolated. A major aim in the field of improvement is therefore to be able to manipulate complex developmental processes genetically in order to improve crop performance. Essential to this objective is the determination of strategies which allow the expression of specific plant genes to be regulated at will.

The ability to control the expression of traits according to circumstances has many important applications, such as control of insect resistance genes, determination of plant height and timing of flowering and the control of plant fertility. Additionally, the ability to switch genes on or off at will, without disturbing plant

physiology or environment, would be an invaluable tool in the study of plant genetics per se.

Currently, the production of seed for hybrid crops such as maize involves the laborious and expensive process of hand or mechanical emasculation of the parent plants in order to prevent self-pollination. Such emasculation can, however, be controlled genetically by making use of a trait known as cytoplasmic male sterility (CMS) which has been observed in a wide variety of crop species. CMS interferes with male gametogenesis, resulting in the inhibition of pollen formation, but does not normally affect female fertility. Consequently, "male-sterile" plants are able to set seed, such seed resulting only from cross-pollination. The ability to control the expression of these genes would allow male gametogenesis to be inhibited in the production of hybrid crop seeds without the need for expensive emasculation processes, while still allowing genetic improvement of the male parent by conventional breeding programmes.

Control of gene expression in both prokaryotes and eukaryotes relies primarily on the interaction of regulatory proteins with specific DNA sequences. Depending on the nature of these interactions, transcription from the cognate promoters may either be repressed or activated. Indeed, in some cases the same protein may either reduce or enhance transcription according to the nature of the contacts made. Furthermore, the ability of some regulatory proteins to bind their target sequences is modulated by the binding of ligands or by

specific proteolytic cleavage. Such mechanisms may be exploited in order to include inducibility amongst strategies for plant gene regulation.

The best characterised regulatory systems are those of bacteria in which the interactions between the DNA-binding proteins (repressors) and the target DNA sequences (operators) are understood in great detail. A comparison of the best understood systems, including repressor and cro proteins of bacteriophage λ and 434, the LacI repressor and the catabolite gene-activating protein (CAP), reveals several factors in common. These regulatory proteins bind as dimers or tetramers to short operators that exhibit a high degree of dyad symmetry. In most cases the domain responsible for DNA-recognition, which is separate from that concerned with oligomerisation of the monomers, contains a conserved helix-turn-helix structure. A specific helix within this structure in each monomer, the recognition helix, is aligned with the major groove of the DNA and only if specific contacts are formed between the amino acids of this recognition helix and the bases of the adjacent DNA can a functional repressor/operator complex be formed. Such interactions are highly specific, and the high-affinity complexes are formed with extremely rapid kinetics.

The knowledge of mechanisms by which gene expression is regulated in eukaryotes is much less detailed. In yeast and mammalian cells a large number of binding sites for putative regulatory proteins have been identified in promoter sequences, and in some cases the proteins responsible have also been isolated. However, only

in a few instances are the molecular details known of the protein-DNA interactions and th mechanism by which transcription is regulated.

In plants, regulation of gene expression is understood at only a rudimentary level. Several regulatory elements have been identified in promoter sequences, and some regulatory proteins examined at a preliminary level. However, such proteins have yet to be isolated and the details of the mechanisms involved elucidated.

Eukaryotic regulatory systems appear to exhibit a greater diversity of structure and a higher degree of complexity than their prokaryotic counterparts. For instance, control of transcription from eukaryotic promoters is thought to involve the interaction of many proteins (perhaps in the order of tens) with the regulatory DNA. Furthermore, at least three different protein structures (the helix-turn-helix, the zinc-finger and the leucine zipper) have been implicated in the specificity of DNA-binding by various eukaryotic regulatory factors.

DNA-binding protein constitute a class of proteins characterised by their ability to bind to DNA of genes to give the effect of either repressing or activating the gene to which they bind. Unless the context otherwise requires, such DNA-binding proteins are hereinafter referred to for convenience simply as "repressors" .

An object of the present invention is to provide means for the control of expression of specific plant genes.

According to the present invention there is provided a recombinant plant gene comprising a

repressor gene of bacterial origin and a promoter which operates in plants for driving expr ssion of the repressor gene, said gene encoding a repressor protein capable of interaction with an operator sequence associated with a selected target plant gene so that on expression of the repressor protein expression of the target plant gene is inhibited.

The invention also provides stably transformed cells containing the said recombinant plant gene.

The invention further provides a vector, preferably the plasmid, designated p35SlacI, containing the said DNA, which has been deposited in an E.coli, strain TG-2, host with the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 12th December 1988, under the Accession Number NCIB 40092.

In addition, the invention provides a plant transformation vector comprising Agrobacterium tumefaciens, harbouring the plasmid aforesaid.

In a specific embodiment of the invention, a bacterial lacI operator system is utilised to regulate gene expression. Lac repression can be relieved by iso-propyl thiogalactoside (IPTG) and other sugar analogues.

The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and

pollen tube transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

The invention will now be described, by way of illustration, in the following example.

EXAMPLE

(1) Construction of plants expressing the lac repressor

Vectors were constructed which express the lacI gene from either the constitutive CaMV 35S promoter found in vector pJR1 or from the green tissue- specific promoter, the maize CAB promoter. However, the bacterial repressor can be expressed from any plant promoter expressed in other parts of the plant, thus allowing control of plant gene expression in any specific part of the plant.

(1.1) Modification and insertion of the lacI repressor gene into pJR1

The lac repressor ($lacI^{Q}$) is available on plasmid pMJR 156. In order to express this gene in plants, the translation initiation codon (GTG) had to be changed into ATG. In addition it was opportune to create a suitable restriction enzyme cleavage site for cloning of this gene into a plant expression vector. At the 3' end of the lacI there are suitable restriction sites (HindIII and PstI) for insertion into plant expression vectors. In order to create suitable restriction sites at the 5' end, the following experiments had to be performed:

(a) A Cfr 10 restriction site is located at position 134. pMJR was cut with Cfr 10 and a synthetic DNA fragment which reconstitutes the N-terminus of the lacI gene, the altered

translational start codon ATG, a plant c nsensus sequence for efficient translational initiation and a BamHI restriction site were inserted into pJR1. The sequence of this synthetic fragment was:

BamHI consensus

GATCC AACAATGGCT AAACCAGTAACGTTATACGATGTCGCAGAGTAT G

G TTGTTACCGA TTTGGTCATTGCAATATGCTACAGCGTCTCATA CGGCC

Cfr 10

pJR1 was cut with BamHI and PstI. The synthetic fragment described above, and the Cfr10 to PstI fragment containing the lacI gene were ligated together with the cut vector pJR1 under standard conditions. The ligation mix was transformed into E.coli TG-2. Recombinants were selected on kanamycin-containing plates. They were characterised by DNA sequence analysis. The construct was designated p35SlacI.

(b) The PCR (Polymerase Chain Reaction) as described by Saiki et.al., Science, 239, 487-491) was utilised to introduce the changes at the 5′ end of the lacI gene while keeping the sequence at the 3′ end. Two oligonucleotides were hybridised to pMJR 156. The sequence of the oligonucleotides were:

(i) from the 5′ end of the gene

BamHI consensus

GAGAGTCAATTCAGGGT GGATCC AACAATGGCT

AAACCAGTAACGTTATACG

(ii) from the 3′ end of the gene

CGTTGTAAAACGACGGCCAGTGCC

The PCR reaction was carried out under the prescribed conditions. The product was cut with BamHI (at the newly introduced site) and PstI.

The resulting fragment was clones into pJR1 cut with BamHI and PstI. Recombinants were identified by hybridisation and restriction analysis using standard protocols. One of the resulting clones was characterised by DNA sequence analysis.

Both of the methods (a) and (b) gave the same construct, designated p35SlacI. Figure 1 shows the structure of this vector.

(1.2) Replacement of the CaMV 35S promoter with maize CAB promoter

In order to demonstrate the general utility of the Lac repressor/operator system in plants, we have constructed an expression vector which will allow inducible and tissue-specific lacI expression in plants. For this work, we have used the promoter of the gene encoding the light-inducible maize chlorophyll a/b binding protein (CAB).

The construction of this vector was achieved by replacing the CaMV promoter in p35SlacI with the maize CAB promoter, the DNA sequence of which is given in Figure 3 herewith, which is found in vector pCAB48.1. The CaMV promoter was removed by restriction of p35SlacI with EcoRI and BamHI using standard conditions. The CAB promoter was isolated from pCAB48.1 by restriction with XbaI and Sau3A using partial restriction conditions for Sau3A. This promoter fragment was then inserted into the promoter-less p35SlacI. This vector, designated pCABlacI, has been characterised by restriction mapping and DNA sequence analysis.

(1.3) Transformation of tobacco plants

The expression modules from the vectors described above were transferred to BIN19 and then to tobacco using leaf disc transformation following

standard protocols. The plasmids were transferred to Agrobacterium using triparental mating. Agrobacteria were purified, and used in leaf disc transformation experiments. Thirty-seven plants containing the CaMV-lacI expression module and thirty-eight plants containing the BAB-lacI construct were regenerated and analysed for the relative expression of lacI.

1.4 Analysis of transgenic plants for lacI expression

The expression of the lacI gene was monitored using Western analysis of extracted proteins. Extracts were prepared, proteins recovered on polyacrylamide gels and prepared for Western analysis. The analyses confirmed the expression of the lacI gene construct in the transformed plants. Different levels of lacI gene expression were observed in different independent transformants. The results for plants transformed with the CaMV-lacI construct are given in the following Table

TABLE	
Plant Sample	Lac Expression (Band Intensity)
L1	-
L2	++
L3	-
L5	-
L7	-
L8	+++
L9	+
L10	+
L11	-
L13	+
L14	++
L15	-
L16	+++
L18	-
L22	+/-
L23	-
L24	++
L25	+
L26	+++
L27	+++
L28	-
L29	++++++
L31	++
L32	++++
L33	+/-
L34	+/-
L35	++
L37	+
- → ++++++ indicates band intensity of Lac repressor from Western blots.	

2. Insertion of the lac operator into target genes

(a) The maize CAB promoter

The maize CAB promoter can be found in plasmid pCAB48.1 and we have found that this promoter can drive expression of foreign genes in a transient tobacco expression system and in stably transformed

plants. This gene, therefore, is an excellent target to demonstrate control through lacI as high levels of expression can be obtained both in vitro and in vivo. Secondly, the CAB promoter from other systems (wheat, pea and tobacco) have been extensively analysed in detail and reported in the literature. The published information facilitates the selection of suitable sites for operator insertion. Thirdly, pCAB48.1 is a maize promoter and the use of this system is important to demonstrate the applicability of this invention to monocotyledonous plants such as maize,wheat, barley and sorghum.

(b) Insertion of the lac operator into the maize CAB promoter

Relatively little work has been reported concerning the characterisation of the important cis-acting elements of the CAB promoter. Therefore, a computer search comparing consensus upstream regulatory elements (UREs) of several plant genes against the CAB promoter was carried out. As anticipated, numerous putative UREs were found in both strands of the CAB promoter. A number of potential sites for operator insertion were selected.

1. 5' of the CAAT box;
2. Between the CAAT and the TATA box;
3. Around the TATA box;
4. Between the TATA box and the transcription start point; and,
5. Between the transcription start point and the translation start point.

Two methods were used to insert the lac operator into the maize CAB promoter:

(1) insertion into naturally occurring restriction sites;

(2) using PCR to introduce operators at selected sites.

These methods were used to insert lacI operators into the selected sites.

Method (1)

Analysis of the promoter sequence shows that this region does not contain many unique restriction sites. However, two sites can be made available by simply recloning the promoter region into various vectors.

(a) Insertion between TATA and TSP

The restriction enzyme PvuII recognises a single site within the 2.8 kb PstI fragment containing the CAB gene. The site lies between the TATA element and the transcription start point (TSP) of the CAB promoter. However, the vector pCAB48.1 contains numerous PvuII sites (within pUC19). Therefore, the 2.8 kb PstI fragment was cloned into the standard cloning vector pAT153 (which lacks a PvuII site) to give pCABP1.

Operator sequences were inserted into the unique PvuII site within pCABP1. After sequencing, it was possible to determine which clones contain single and tandem operator insertions. The synthetic symmetric lac operator required for this work is shown below and is an 18 base pair palindrome that is analogous to a mutant operator which binds lac repressor eight times more strongly than the wild-type operator.

lac operator-1

5′ -ATTGTGAGCGCTCACATT- 3′

(b) Insertion upstream of the CAAT sequence

The method used was as follows:

(i) pCAB48.1 was digested with HindIII, which cuts outside the promoter region and within pUC18, and BglII, which cuts downstream of the unique NcoI site and within the coding region. This gave a fragment with a unique SphI site upstream of the CAAT moiety;

(ii) pUC18 was digested with HindIII and BamHI and the promoter fragment from (i) above was inserted to give pCABP2. Digestion of pUC18 with BamHI removes the single SphI site from the polylinker. Therefore, pCABP2 contains a unique SphI site into which operators can be inserted.

The operator used in this procedure had the sequence:

lac Operator-2

5'-ATTGTGAGCGCTCACAATCAT G-3'

3'-GTACTAACTCTCGCGAGTGTTA-5'

It is important to note that in approaches (a) and (b) the operator sequences are not inserted directly into any putative regulatory elements although promoter activity is likely to be affected when the sequences are inserted elsewhere.

Method 2

As shown above, operator sequences can be inserted into two available restriction sites. Insertion into other sites requires other methodologies.

Insertion between TSP and ATG codon

This can be effected using PCR. Since a unique PvuI site lies close to the TSP region, it is used as a reference point for subcloning purposes. The starting material for the PCR reaction is pCABP1, that is, the pAT 153 CAB

promoter vector constructed as described above. An oligonucleotide overlapping the PvuII site and containing no alterations was used to prime the reaction from one end:

CAB Oligonucleotide-1

PvuII

5'-GG CAGCTG CTGTGTTCTGTTATGAC-3'

The second oligonucleotide overlaps the NcoI site and contains the operator sequence shown below.

CAB Oligonucleotide-2

NcoI Operator 1

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT

--ATCAGATCGTAGCTCCTTCTGATGC-3'

CAB Oligonucleotide-3

NcoI Operator 1

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT

Operator 2

-ATTGTGAGCGCTCACAAT ATCAGATCGTAGCTCCTTCTGATGC-3'

Following the PCR reactions, the newly synthesised DNA is cleaved with PvuII and NcoI. The fragment is then transferred to similarly digested pCABP1 and sequenced.

A slightly different approach which eliminates the intermediate cloning step into pCABP1 may also be used. This involves using an oligonucleotide which overlaps the unique XbaI site in the CAB promoter together with the operator nucleotides outlined previously. Digestion of PCR DNA with XbaI/NcoI results in a fragment which can be directly cloned into pCG1 and pCG2. However the XbaI to NcoI fragment from the PCR reaction is much larger than the PvuII to NcoI fragment obtained from the previous strategy.

Op rator insertion between the CAAT and TATA

This is effected using PCR.

CAB Nucleotide-4

XbaI

5'-CCCAAACAG TCTAGA TATGTTTCTC-3'

CAB Nucleotide-5

PvuII Operator

5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-
-AGTTGGGTTTGGATAGCAGGTCATC-3'

CAB Nucleotide-6

PvuII Operator 1

5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-
Operator 2
-ATTGTGAGCGCTCACAAT AGTTGGGTTTGGATAGCAGGTCATC-3'

Following PCR, DNA is digested with XbaI and PvuI and cloned into similarly digested pCABP1. Clones are again characterised by sequencing and any appropriate DNAs are digested with XbaI and NcoI and cloned into pCG1 and pCG2. The basic structure of these vectors is shown in Figure 2.

The CAMV 35S promoter

We have found that a promoter-less 35S vector is an excellent receptor for the insertion of activating sequences. The lac operator can be inserted into this vector, p-Δ-35S, and once inserted the 35S enhancer is cloned 5' upstream of the lac operator.

(3) Control of gene expression by lac repressor

(a) Control of target gene expression in a transient expression system

Plants which express lacI constitutively transformed with p35lacI may be prepared from protoplasts and, using methods described above) they may be tested for expression of the lacI protein. The target gene constructs may then be introduced

into the protoplasts using standard methods and protocols. Protoplasts from untransformed plants can s rve as control. Further control may be provided by protoplasts from plants expressing the GUS marker gene under the control of the CAB promoter without the operator insertions.

(b) Induction of gene expression using IPTG

IPTG can be used to overcome repression by the lac repressor. Thus, there is formed a switchable gene system.

(c) Modulation of expression of the target gene

Lac repressor/operator interactions can down-regulate marker gene expression in plants to different levels. This is an important effect in that there may be situations where a different degree of down-regulation may be required.

(d) Control of target gene expression in stably transformed plants

Having shown, as described above, that the lac-repressor can down-regulate CAB promoter driven GUS expression in protoplasts , suitable operator insertion constructs may be transferred to tobacco plants by the methods described above. The regenerated plants may be crossed with the lacI expressing plants described above, which express the lac repressor under control of the constitutive CaMV35S promoter.

Plants may also be constructed which express the lacI gene under control of the light-inducible maize CAB promoter. The expression of the lacI gene in these plants will then be light-inducible. These plants may be crossed with plants which contain the GUS marker gene from the CaMV promoter containing the lacI operator insertion.

Insertion of multiple operators into the CAB promoter

Using similar techniques as described for insertion of single operators, multiple operators can be inserted into the target promoter. This can either be by the insertion of multiple copies of the operator at one site, or the combination of fragments of the promoter in which the operator is inserted at different positions in the promoter, this yielding vectors in which the multiple operators are located at multiple locations in the promoter.

Claims

1. A recombinant plant gene comprising a DNA-binding protein gene and a promoter which operates in plants for driving expression of the said DNA-binding protein gene, said DNA-binding protein gene being capable of interaction with an operator sequence associated with a selected target plant gene.

2. A recombinant gene as claimed in claim 1, in which the DNA-binding protein gene encodes a repressor protein so that on expression of the DNA-binding protein gene expression of protein by the target plant gene is inhibited.

3. A recombinant gene as claimed in claim 1 or claim 2 in which both the DNA-binding protein gene and the said operator are of non-plant origin.

4. A recombinant gene as claimed in claim 1 or claim 2 in which both the DNA-binding protein gene and the said operator are of bacterial origin.

5. A recombinant gene comprising (a) a DNA-binding protein gen and a promoter which operates in plants for driving expr ssion of the said DNA-binding protein gene, said DNA-binding protein gene being capable of interaction with an operator sequence associated with a selected target plant gene, and (b) operatively linked to the said operator, a plant gene encoding a protein conferring a selected plant characteristic when expressed in a host plant.

6. A recombinant gene as claimed in claim 1 or claim 2, in which the repressor gene and the operator are derived from the lacI gene of Escherichia coli.

7. A plasmid, designated p35SlacI, containing the said DNA, which has been deposited in an Escherichia coli, strain TG-2, host with the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 12th December 1988, under the Accession Number NCIB 40092.

8. A plant transformation vector comprising Agrobacterium tumefaciens, harbouring the plasmid claimed in claim 4.

9. A plant transformed with the vector claimed in claim 5.

10. A plant having stably incoporated in its genome a recombinant gene claimed in any of claims 1 to 3.

FIG. 1. Cloning PCR-amplified lacI

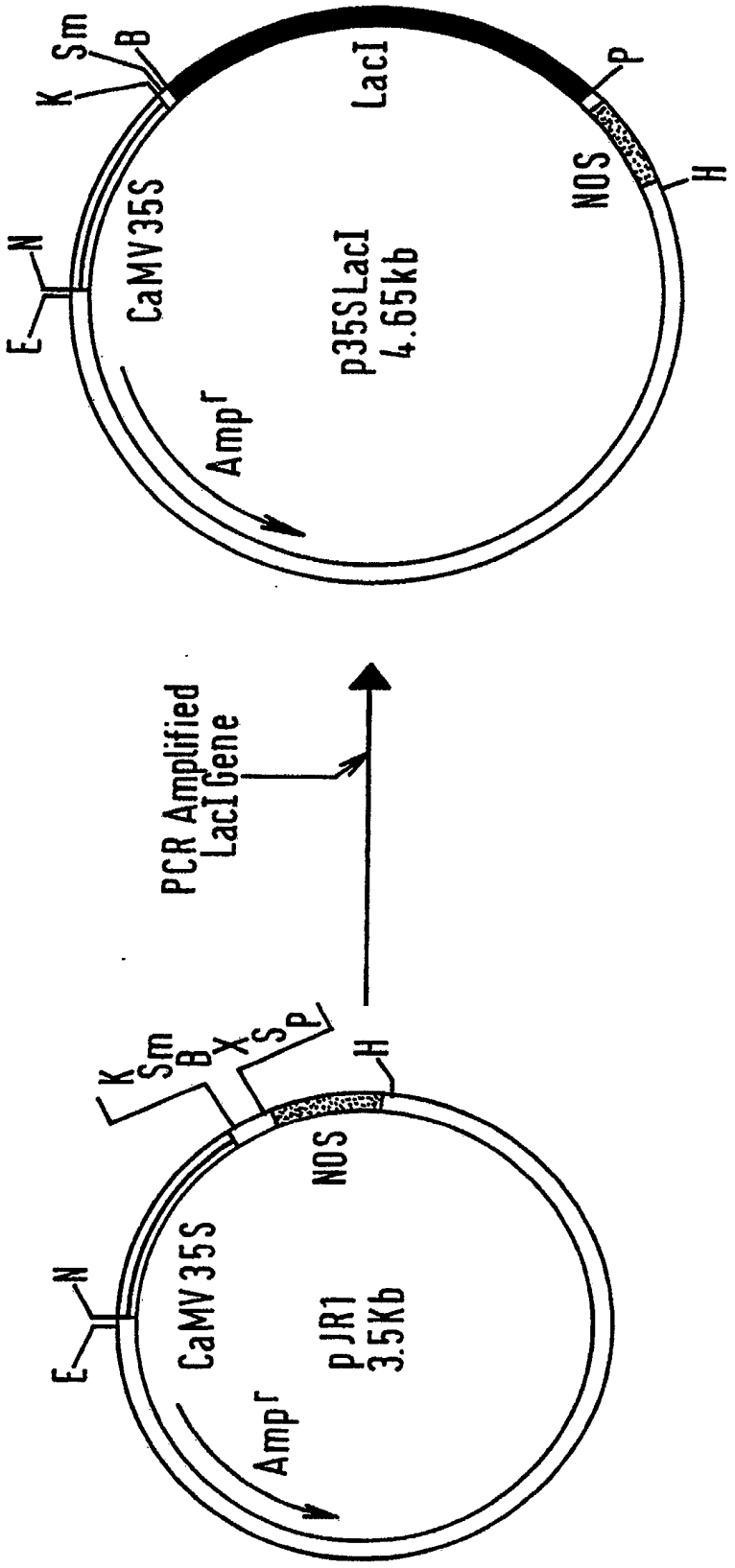
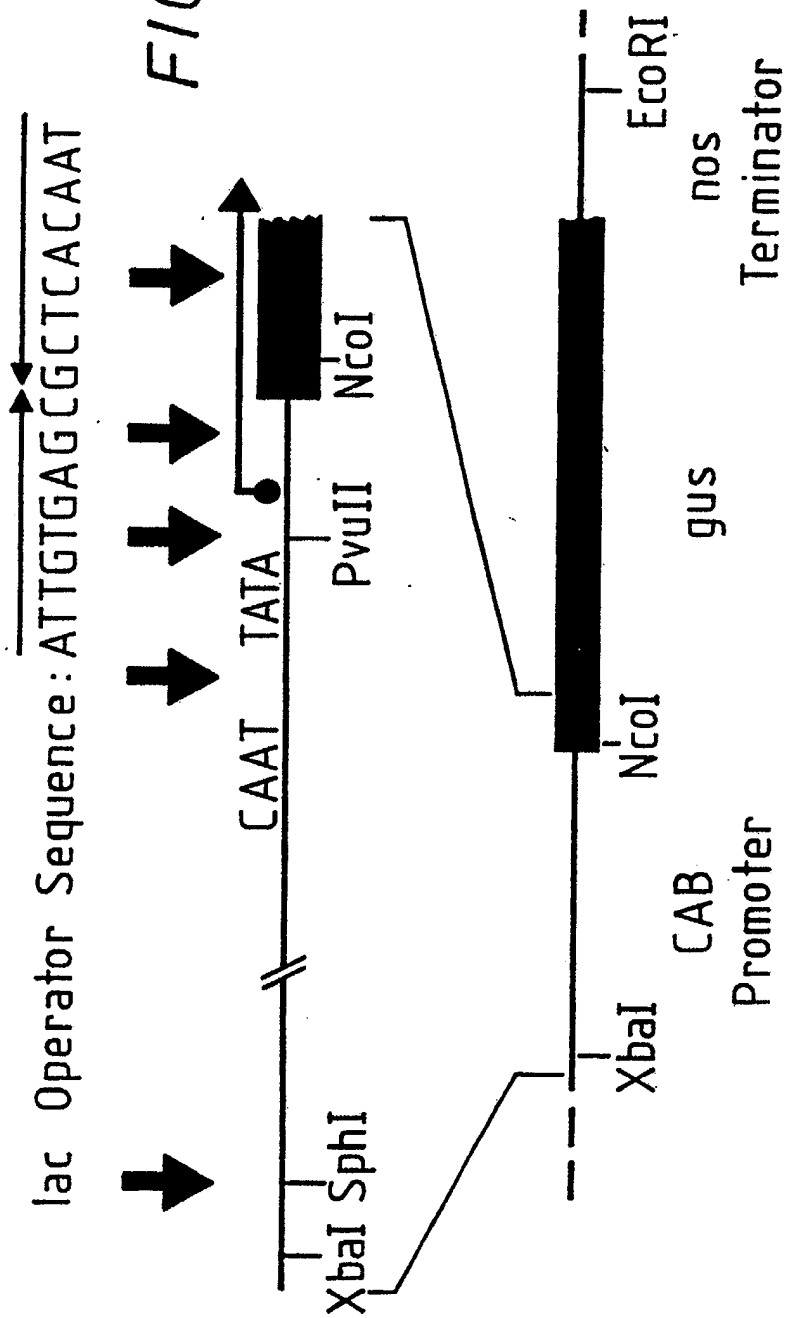


FIG.2.


lac Operator Sequence: ATTGTGAGCGCTCACAAT
CAAT
TATA
PvuII
NcoI
XbaI
SphI
NcoI
XbaI
CAB
Promoter
gus
nos
Terminator
EcoRI

SEQUENCE OF THE XbaI - NcoI CAB PROMOTER FRAGMENT.

```
                10         20         30         40         50
    XbaI        :          :          :          :          :
   1 GTCTAGATATGTTTCTCTTTTGGTTCTTGAGTTGACAAGTGGCATGCTAT
  51 TTTTGCTCATGTGAGAAAAAATATAGCACCGTTTGTTTCGTTGAAATTGA
 101 ATTGCATCCCAATAATCATAATTTAGACATAAACCAACTAAGTTAATATA
 151 TTTGTATATGTAAATATGTTATCCTAAATTATAATTATATGAGAGAGATA
 201 GTTATACATTACAATTATGATATAGAGAAGCAAATAGAAGAGTGTGCTAT
 251 AAGTTGTACATTGTAAAAGTAGTATGCAAATTTATAGAATTAATTTTTAT
 301 CTTTCACCTCATAAATTTAAGATACACTTATATATAAACTTTGAAAAGTT
 351 GTAAAATATCATATTCTAAAAAAAATAGACTATTCTATTAGTTAGATTCC
 401 AATTCCTTAAAATAAAAGAAAATAAATGGGCTTAAGGGCTAGTTTGGTGG
 451 GCAGGTGGAGGCGATCAATGGCGGATGAATCCTCTCGATATTCAATTGAG
 501 GGGGTTCATCCCATGAATCTCTCTATTTACATGTCCACCAAACAAGATTA
 551 AATGCAATGATACATAGCTTTTGTGCATATTTTTTTTTAACGGAGATGCA
 601 AAACAGTATACAAGATGTGTGTATGTTCCCATGCGACCCATCTTGTATCC
 651 AGTTCATGCGTCTGGTGGTTGAACCAGAAAGATACACATGATCCATTTCG
 701 ATGAATCAACACAAAAGACTTCATTGATTATGCATTTGACAAATAGCAAG
 751 AAGCGCGGGGCAACAAAGCCCATCCTATCTCATCCATTTTTTCCCACGAT
 801 GGCAAGTGGCAGCTCCTGATTAGCTACGCCCATTCCTATGCTATGTGGCA
 851 CACCCCAGGATTCTTGTGTGATAGGCCATTGGGGCCACGAGGAGCCACGT

 901 CAGACGCCAAGCCACCCCGGCGAGACCAACCAGCCAATCGCAGTTCAGGA

 951 AAAGATGACCTGCTATCCAAACCCAACTGTATAAAAGGCAGCTGCTGTGT
               * * * *
1001 TCTGTTATGACACAGCCATCACACGCATACTGCATACACAACACAGAGCA
                                     M  A  A  A  T  M
1051 TCAGAAGGAGCTACGATCTGATCGACATGGCTGCCGCCACCATGG
                                                 NcoI
```

_____ = CAT and TATA sequences.

* = Possible transcription start sites.

FIG. 3.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00102

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, A 01 H 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[5]	C 12 N, A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proc. Natl. Acad. Sci. USA, volume 85, March 1988, C. Gatz et al.: "Tn10-encoded tet repressor can regulate an operator-containing plant promoter", pages 1394-1397 see the whole article	1-5,8-10
Y	--	6
O,P, X	Journal of Cellular Biochemistry, Suppl 13D, Plant Gene Transfer, 1-7 April 1989, Alan R. Liss, Inc., (New York, US), A. Merryweather et al.: "Control of plant gene expression using wild-type and altered-specificity bacterial repressor molecules", page 307, abstract M 334 see the abstract	1-10
Y	-- Cell, volume 48, 27 February 1987, Cell Press, ./.	6

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd May 1990	- 5. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

International Application No. PCT/GB 90/00102

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC ND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	M.C.-T. Hu et al.: "The inducible lac operator-repressor system is functional in mammalian cells", pages 555-566 see the whole article	